

Gene encoding thanatin, vector containing it and
disease-resistant transformed plants obtained

The subject of the present invention is a DNA
5 sequence encoding thanatin, a vector containing it for
the transformation of a host organism, and the method
of transforming the said organism.

The invention relates more particularly to
the transformation of plant cells and plants, the
10 thanatin produced by the transformed plants conferring
on them resistance to diseases, in particular of fungal
origin.

An increasing need already exists for making
plants resistant against diseases, in particular fungal
15 diseases, in order to reduce, or even eliminate, the
need for treatment with antifungal protection products,
with a view to protecting the environment. One means of
increasing this resistance to diseases consists in
transforming the plants so that they produce substances
20 capable of providing their defence against these
diseases.

Various substances of natural origin, in
particular peptides, are known which exhibit
bactericidal or fungicidal properties, in particular
25 against the fungi responsible for plant diseases.
However, the problem consists in finding such

substances which not only can be produced by transformed plants, but can still preserve their bactericidal or fungicidal properties and confer them on the said plants. For the purposes of the present
5 invention, bactericidal or fungicidal is understood to mean the actual bactericidal or fungicidal properties and the bacteriostatic and fungistatic properties.

Thanatin is a peptide produced by bacterial induction on adult *Psodius sp*, preferably
10 *maculiventris*. Its preparation by bacterial induction is described in patent application FR 2,733,237, as well as its antifungal and antibacterial properties *in vitro*.

After having first identified the thanatin
15 gene, it was also found that it could be inserted into a host organism, in particular a plant, in order to express the thanatin and confer on the said host organism properties of resistance to fungal diseases and to diseases of bacterial origin, providing a
20 particularly advantageous solution to the problem stated above.

The subject of the invention is therefore first a nucleic acid fragment encoding thanatin, a chimeric gene comprising the said fragment encoding
25 thanatin as well as heterologous regulatory elements at the 5' and 3' positions which can function in a host organism, in particular in plants, and a vector for transforming the host organisms containing this

chimeric gene, and the transformed host organism. It also relates to a transformed plant cell containing at least one nucleic acid fragment encoding thanatin and a disease-resistant plant containing the said cell, in particular regenerated from this cell. It finally relates to a method of transforming plants to make them resistant to diseases, in which method a gene encoding thanatin is inserted by means of an appropriate vector.

Thanatin is understood to mean according to the invention any peptide comprising essentially the peptide sequence of 11 amino acids which is described in patent application FR 2,733,237, as well as the equivalent homologous sequences in which certain amino acids are replaced by different but equivalent amino acids at sites which do not induce substantial modification of the antifungal or antibacterial activity of the said homologous sequence. Peptide sequence comprising essentially the peptide sequence described in patent application FR 2,733,237 is understood to mean not only the sequence defined by the sequence identifier No. 1 (SEQ ID NO 1), but also such a sequence comprising at either of its ends, or at both, peptide residues necessary for its expression and targeting in a host organism, in particular a plant cell or a plant.

Thanatin is a peptide of formula (I):

Xaa-Ile Ile Tyr Cys Asn Arg Arg Thr Gly Lys Cys-Xab

(I)

(SEQ ID NO:14)

B

in which:

Xaa is NH_2 or a variable residue having a sequence comprising from 1 to 10 amino acids, and

Xab is OH or a variable residue having a
5 sequence comprising from 0 to 5 amino acids.

Advantageously, when Xaa comprises at least one amino acid, the latter is one of the 20 base amino acids and more particularly chosen from the group comprising Gly, Ser, Lys, Pro and Val. When Xab
10 comprises at least one amino acid, the latter is one of the 20 base amino acids and more particularly chosen from the group comprising Gln, Arg and Met.

According to a preferred embodiment of the invention, the two cysteine residues of the peptide of
15 formula (I) form an intramolecular disulphide bridge.

The present invention therefore relates first to a nucleic acid, in particular a DNA, fragment encoding the thanatin defined above. It may be, according to the invention, a fragment isolated from
20 *Psodius sp*, preferably *maculiventris*, or alternatively a derived fragment, suitable for the expression of thanatin in the host organism where the peptide will be expressed. The nucleic acid fragment may be obtained using standard methods of isolation and purification,
25 or alternatively by synthesis according to the customary techniques of successive hybridizations of synthetic oligonucleotides. These techniques are in particular described by Ausubel et al.

According to the present invention, "nucleic acid fragment" is understood to mean a nucleotide sequence which may be of the DNA or RNA type, preferably of the DNA, in particular cDNA, especially double-stranded, type.

According to one embodiment of the invention, the nucleic acid fragment encoding thanatin comprises the DNA sequence described by the sequence identifier No. 1 (SEQ ID NO 1), a homologous sequence or a sequence complementary to the said sequence.

Advantageously, the nucleic acid fragment according to the invention comprises the DNA sequence described by the sequence identifier No. 2 (SEQ ID NO 2), a homologous sequence or a sequence complementary to the said sequence.

"Homologous" is understood to mean according to the invention a nucleic acid fragment having one or more sequence modifications relative to the nucleotide sequence described by the sequence identifier No. 1 or No. 2 and encoding thanatin. These modifications may be obtained according to the customary mutation techniques, or alternatively by choosing the synthetic oligonucleotides used in the preparation of the said sequence by hybridization. Given the multiple combinations of nucleic acids which may lead to the expression of the same amino acid, the differences between the reference sequence described by the sequence identifier No. 1 or No. 2 and the homologue

may be great, especially since a DNA fragment of less than 100 nucleic acids in size, which can be produced by synthesis, is involved. Advantageously, the degree of homology will be at least 70% relative to the
5 reference sequence, preferably at least 80%, more preferably at least 90%. These modifications are generally neutral, that is to say that they do not affect the primary sequence of the resulting thanatin.

The present invention also relates to a
10 chimeric gene (or an expression cassette) comprising a coding sequence as well as heterologous regulatory elements at the 5' and 3' positions which can function in a host organism, in particular plant cells or plants, the coding sequence comprising at least one DNA
15 fragment encoding thanatin as defined above.

Host organism is understood to mean any higher or lower mono- or pluricellular organism into which the chimeric gene according to the invention may be introduced, for the production of thanatin. It
20 consists of in particular bacteria, for example *E. coli*, yeasts, in particular of the genera *Saccharomyces* or *Kluyveromyces*, or preferably plant cells and plants.

"Plant cell" is understood to mean according
25 to the invention any cell derived from a plant and which may constitute undifferentiated tissues such as calli, and differentiated tissues such as embryos, plant portions, plants or seeds.

5 cultivated plants intended or otherwise as animal feed
or for human consumption, such as maize, wheat, colza,
soya bean, rice, sugar cane, beet, tobacco, cotton and
the like.

10 expression of the DNA fragment encoding thanatin are well known to persons skilled in the art depending on the host organism. They comprise in particular promoter sequences, transcription enhancers, transit peptides, terminator sequences, including start and stop codons.

15 The means and methods for identifying and selecting the regulatory elements are well known to persons skilled in the art.

20 fused in 5' and/or 3' to the sequence encoding thanatin,
so as to obtain a "protein-thanatin" fusion protein,
whose cleavage by the enzymatic systems of the host
organism allows the release of thanatin. This thanatin-
fused protein may be a signal peptide or a transit
25 peptide which makes it possible to control and orient
the production of thanatin in a specific manner in a
part of the host organism, such as for example the
cytoplasm, the cell membrane, or in the case of plants

in a particular type of tissue or in the extracellular matrix.

According to one embodiment, the transit peptide may be a signal for chloroplast or
 5 mitochondrial addressing, which transit peptide is then cleaved in the chloroplast or the mitochondria.

According to another embodiment of the invention, the signal peptide may be an N-terminal signal or "prepeptide", optionally in combination with
 10 a signal responsible for retaining the protein in the endoplasmic reticulum, or a peptide for vacuolar addressing or "propeptide". The endoplasmic reticulum is the site where operations of maturation of the protein produced, such as for example the cleavage of
 15 the signal peptide, are carried out by the "cellular machinery".

The invention relates more particularly to the transformation of plants. As regulatory promoter sequence in plants, there may be used any promoter
 20 sequence of a gene which is expressed naturally in plants, in particular a promoter of bacterial, viral or plant origin such as, for example, that of a gene for the small subunit of ribulose biscarboxylase (RuBisCO) or of a plant virus gene, for example that of
 25 cauliflower mosaic (CAMV 19S or 35S), or a promoter inducible by pathogens such as tobacco PR-1a or asparagus AoPRT-L, it being possible for any known suitable promoter to be used. Preferably, a regulatory

promoter sequence is used which promotes the overexpression of the coding sequence constitutively or inducibly by a pathogen attack, such as for example that comprising at least one histone promoter as
5 described in application EP 0,507,698.

According to the invention, it is also possible to use, in combination with the regulatory promoter sequence, other regulatory sequences which are situated between the promoter and the coding sequence,
10 such as transcription enhancers such as for example the tobacco mosaic virus (TMV) translation enhancer described in application WO 87/07644, or the tobacco etch virus (TEV) translation enhancer described by Carrington & Freed, or transit peptides, either single
15 or double, and in this case optionally separated by an intermediate sequence, that is to say comprising, in the direction of transcription, a sequence encoding a transit peptide of a plant gene encoding a plastid localization enzyme, a portion of sequence of the N-
20 terminal mature portion of a plant gene encoding a plastid localization enzyme, and then a sequence encoding a second transit peptide of a plant gene encoding a plastid localization enzyme consisting of a portion of sequence of the N-terminal mature portion of
25 a plant gene encoding a plastid localization enzyme, as described in application EP 0,508,909. As transit peptide, there may be mentioned the signal peptide of the tobacco PR-1a gene described by Cornelissen et al.,

represented with its coding sequence by the sequence identifier No 3.

The sequence encoding the fusion protein signal peptide PR-1a-thanatin and this fusion protein
5 also form part of the present invention. This sequence is in particular described by the sequence identifier No. 5, more particularly the coding part of this sequence, corresponding to bases 12 to 164.

As regulatory terminator or polyadenylation
10 sequence, there may be used any corresponding sequence of bacterial origin, such as for example the nos terminator from *Agrobacterium tumefaciens*, or alternatively of plant origin, such as for example a histone terminator as described in application
15 EP 0,633,317.

According to the present invention, the chimeric gene may also comprise a selectable marker suitable for the transformed host organism. Such selectable markers are well known to persons skilled in
20 the art. They may be a gene for resistance to antibiotics, such as penicillin, or alternatively a gene for tolerance of herbicides for plants.

The present invention also relates to a cloning or expression vector for the transformation of
25 a host organism containing at least one chimeric gene as defined above. This vector comprises, in addition to the above chimeric gene, at least one replication origin. This vector may consist of a plasmid, a cosmid,

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a bacteriophage or a virus, transformed by the introduction of the chimeric gene according to the invention. Such transformation vectors, depending on the host organism to be transformed, are well known to persons skilled in the art and are widely described in the literature.

For the transformation of plant cells or plants, they may consist in particular of a virus which may be used for the transformation of developed plants and containing, in addition, its own elements for replication and expression. Preferably, the vector for transforming plant cells or plants according to the invention is a plasmid.

The subject of the invention is also a method of transforming host organisms, in particular plant cells, by integration of at least one nucleic acid fragment or a chimeric gene as defined above, which transformation may be obtained by any known appropriate means widely described in the specialist literature and in particular the references cited in the present application, more particularly by the vector according to the invention.

A series of methods consists in bombarding cells or protoplasts with particles to which the DNA sequences are attached. Another series of methods consists in using, as means of transferring into the plant, a chimeric gene inserted into a Ti plasmid from

Agrobacterium tumefaciens or an Ri plasmid from *Agrobacterium rhizogenes*.

Other methods may be used, such as microinjection or electroporation or alternatively
5 direct precipitation by means of PEG.

Persons skilled in the art will make the choice of the appropriate method depending on the nature of the host organism, in particular the plant cell or the plant.

10 The subject of the present invention is also the host organisms, in particular plant cells or plants, transformed and containing an effective quantity of a chimeric gene comprising a sequence encoding the thanatin defined above.

15 The subject of the present invention is also the plants containing transformed cells, in particular the plants regenerated from transformed cells. The regeneration is obtained by any appropriate method which depends on the nature of the species, as for
20 example described in the references above.

For the methods of transforming plant cells and of regenerating plants, the following patents and patent applications may be mentioned: US 4,459,355, US 4,536,475, US 5,464,763, US 5,177,010, US 5,187,073,
25 EP 267,159, EP 604 662, EP 672 752, US 4,945,050, US 5,036,006, US 5,100,792, US 5,371,014, US 5,478,744, US 5,179,022, US 5,565,346, US 5,484,956, US 5,508,468, US 5,538,877, US 5,554,798, US 5,489,520, US 5,510,318,

US 5,204,253, US 5,405,765, EP 442,174, EP 486,233,
EP 486,234, EP 539,563, EP 674,725, WO 91/02071 and
WO 95/06128.

The present invention also relates to the
5 transformed plants derived from the cultivation and/or
crossing of the above regenerated plants, as well as
the seeds of transformed plants.

The plants thus transformed are resistant to
certain diseases, in particular to certain fungal or
10 bacterial diseases. Consequently, the DNA sequence
encoding thanatin may be integrated with the main
objective of producing plants resistant to the said
diseases, thanatin being effective against fungal
diseases such as those caused by *Cercospora*, in
15 particular *Cercospora beticola*, *Cladosporium*, in
particular *Cladosporium herbarum*, *Fusarium*, in
particular *Fusarium culmorum* or *Fusarium graminearum* or
by *Phytophthora*, in particular *Phytophthora cinnamomi*.

The chimeric gene may also advantageously
20 comprise at least one selectable marker, such as one or
more herbicide tolerance genes.

The DNA sequence encoding thanatin may also
be integrated as a selectable marker during the
transformation of plants with other sequences encoding
25 other peptides or proteins of interest such as, for
example, herbicide tolerance genes.

Such herbicide tolerance genes are well known
to a person skilled in the art and are in particular

described in patent applications EP 115 673,
WO 87/04181, EP 337 899, WO 96/38567 or WO 97/04103.

Of course, the transformed cells and plants
according to the invention may comprise, in addition to
5 the sequence encoding thanatin, other heterologous
sequences encoding other additional peptides capable of
conferring on the plant resistance to other diseases of
bacterial or fungal origin.

The other sequences may be integrated by
10 means of the same vector comprising a chimeric gene,
which comprises a first sequence encoding thanatin and
at least one other sequence encoding another peptide or
protein of interest.

They may also be integrated by means of
15 another vector comprising at least the said other
sequence, according to the customary techniques defined
above.

The plants according to the invention may
also be obtained by crossing parents, one carrying the
20 gene according to the invention encoding thanatin, the
other carrying a gene encoding at least one other
peptide or protein of interest.

Among the sequences encoding other antifungal
peptides, there may be mentioned that encoding
25 drosomycin, which is described in patent application FR
2,725,992 and by Fehlbaum et al. (1994), and in the
unpublished patent application FR 97 09115 filed on
24 July 1997, or that encoding androctonin described in

patent application FR 2,745,004 and in unpublished patent application FR 97 10362 filed on 20 August 1997.

The examples below make it possible to illustrate the invention, the preparation of the sequence encoding thanatin, of the chimeric gene, of the integration vector and of the transformed plants. Figures 1 to 5 in the annex describe the schematic structures of some plasmids prepared for the construction of chimeric genes. In these figures, the different restriction sites are marked in italics.

Example 1: Construction of the chimeric genes

All the techniques used below are standard laboratory techniques. The detailed protocols of these techniques are in particular described in Ausubel et al.

pRPa-MD-P: Creation of a plasmid containing the signal peptide of the tobacco PR-1a gene

The two complementary synthetic oligonucleotides Oligo 1 and Oligo 2 below are hybridized at 65°C for 5 minutes and then by slowly reducing the temperature to 30°C for 30'.

(SEQ ID NO:6)

25 Oligo 1: 5' GCGTCGACGC GATGGGTTTC GTGCTTTTCT CTCAGCTTCC
 ^
 ATCTTTCCTT CTTGTGTCTA CTCTTCTTCT TTTCC 3'

B (SEQ ID NO: 7)
 Oligo 2: 5' TCGCCGGCAC GGCAAGAGTA AGAGATCACA AGGAAAAGAA
 A
 GAAGAGTAGA CACAAGAAGG AAAGATGGAA GC 3'

After hybridization between Oligo 1 and Oligo
 5 2, the DNA remaining single-stranded serves as a
 template for the Klenow fragment of polymerase I of *E.*
coli (under the standard conditions recommended by the
 manufacturer (New England Biolabs)) for the creation of
 the double-stranded oligonucleotide starting from the 3'
 10 end of each oligo. The double-stranded oligonucleotide
 obtained is then digested with the restriction enzymes
SacII and *NaeI* and cloned into the plasmid pBS II SK(-)
 (Stratagene) digested with the same restriction
 enzymes. A clone is then obtained comprising the region
 15 encoding the signal peptide of the tobacco PR-1a gene
 (SEQ ID NO 3).

**pRPA-PS-PR1a-tha: Creation of a sequence encoding
 thanatin fused to the signal peptide PR-1a without a
 20 nontranscribed region in 3'**

The two synthetic oligonucleotides with
 complementary sequences Oligo 3 and Oligo 4 based on
 the operating conditions described for **pRPA-MD-P**.

B (SEQ ID NO: 9)
 25 Oligo 3: 5' GGTTCCAAGA AGCCAGTGCC AATCATCTAC TGCAACAGGA CG 3'
 A

(SEQ ID NO: 9)

Oligo 4: 5' CCGGATCCGT CGACACGTTT GCCTCGCCGA GCTCACATCC
 TCTGGCACTT ACCAGTCCTC CTGTTGCAGT AGATGATTGG
 CACTGGC 3'

5 After hybridization between Oligo 3 and Oligo
 4, the DNA remaining single-stranded serves as a
 template for the Klenow fragment of polymerase I of *E.*
coli (under the standard conditions recommended by the
 manufacturer (New England Biolabs)) for the creation of
 10 the double-stranded oligonucleotide starting from the 3'
 end of each oligo. This double-stranded oligonucleotide
 containing the coding part of thanatin (SEQ ID NO 1) is
 then directly cloned into the plasmid pRPA-MD-P which
 has been digested with the restriction enzyme *NaeI*. The
 15 correct orientation of the clone obtained is checked by
 sequencing. A clone is then obtained comprising the
 region encoding the fusion protein PR-1a-thanatin
 situated between the *NcoI* restriction sites at the N-
 terminal end and the *ScaI*, *SacII* and *BamHI* restriction
 20 sites at the C-terminal end (SEQ ID NO 4).

pRPA-RD-229: Creation of an expression vector in plants
comprising the sequence encoding the fusion protein PR-
1a-thanatin

25 The plasmid pRTL-2 GUS, derived from the
 plasmid pUC-19, was obtained from Dr Jim Carrington
 (Texas A&M University, not described). This plasmid,
 whose schematic structure is represented in Figure 1,

contains the double CaMV 35S promoter isolated from the cauliflower mosaic virus (CaMV 2x35S promoter, Odell et al., 1985) which directs the expression of an RNA containing [lacuna] tobacco etch virus 5' untranslated sequence (TEV 5' UTR; Carrington & Freed, 1990), the *E.coli* β -glucuronidase gene (GUS; Jefferson et al., 1987) followed by the polyadenylation site of the CaMV 35S RNA (CaMV polyA; Odell et al., 1985).

The plasmid pRTL-2 GUS is digested with the restriction enzymes *NcoI* and *BamHI*, and the large DNA fragment is purified. The plasmid pRPA-PS-PR1a-than is digested with the restriction enzymes *NcoI* and *BamHI*, and the small DNA fragment containing the region encoding the fusion protein PR-1a-thanatin is purified. The two DNA fragments purified are then ligated together in an expression cassette in plants which synthesizes a PR-1a-thanatin fusion protein. The schematic structure of this expression cassette is represented in Figure 2. "PR-1a-thanatin" represents the coding region for the fusion protein PR-1a-thanatin of pRPA-RD-230. The thanatin is transported to the extracellular matrix of the plant by the action of the signal peptide PR-1a.

25 **pRPA-RD-195: Creation of a plasmid containing a modified multiple cloning site**

The plasmid pRPA-RD-195 is a plasmid derived from pUC-19 which contains a modified multiple cloning

site. The complementary synthetic oligonucleotides Oligo 5 and Oligo 6 below are hybridized and made double-stranded according to the procedure described for pRPA-MD-P.

5

(SEQ ID NO:10)

Oligo 5: 5' AGGGCCCCCT AGGGTTTAAA CGGCCAGTCA GGCCGAATTC
 GAGCTCGGTA CCCGGGGATC CTCTAGAGTC GACCTGCAGG
 CATGC 3'

(SEQ ID NO:11)

10 Oligo 6: 5' CCCTGAACCA GGCTCGAGGG CGCGCCTTAA TTTAAAGCTT
 GCATGCCTGC AGGTCGACTC TAGAGG 3'

The double-stranded oligonucleotide obtained is then ligated into pUC-19 which has been previously digested with the restriction enzymes *EcoRI* and *HindIII* and made blunt ended using the Klenow fragment of DNA polymerase I of *E. coli*. A vector is obtained containing multiple cloning sites in order to facilitate the introduction of expression cassettes into an *Agrobacterium tumefaciens* vector plasmid. The schematic structure of this multiple cloning site is represented in Figure 3.

pRPA-RD-232: Introduction of the PR-1a-thanatin

expression cassette of pRPA-RD-229 into pRPA-RD-195

The plasmid pRPA-RD-230 is digested with the restriction enzyme *HindIII*. The DNA fragment containing the PR-1a-thanatin expression cassette is purified. The

purified fragment is then ligated into pRPA-RP-195 which has previously been digested with the restriction enzyme *HindIII* and dephosphorylated with calf intestinal phosphatase.

5

pRPA-RD-174: Plasmid derived from pRPA-BL-150A (EP 0,508,909) containing the bromoxynil tolerance gene of pRPA-BL-237 (EP 0,508,909)

The bromoxynil tolerance gene is isolated from pRPA-BL-237 by a PCR gene amplification. The fragment obtained is blunt-ended and is cloned into the *EcoRI* site of pRPA-BL-150A which has been made blunt-ended by the action of Klenow polymerase under standard conditions. An *Agrobacterium tumefaciens* vector is obtained which contains the bromoxynil tolerance gene close to its right border, a kanamycin tolerance gene close to its left border and a multiple cloning site between these two genes.

The schematic structure of pRPA-RD-174 is represented in Figure 4. In this figure, "nos" represents the *Agrobacterium tumefaciens* nopaline synthase polyadenylation site (Bevan et al., 1983), "NOS pro" represents the *Agrobacterium tumefaciens* nopaline synthase promoter (Bevan et al., 1983), "NPT II" represents the neomycin phosphotransferase gene of the *E. coli* Tn5 transposon (Rothstein et al., 1981), "35S pro" represents the 35S promoter isolated from the cauliflower mosaic virus (Odell et al., 1985), "BRX"

3

Author	Year	Country	Sample Size	Study Design	Findings
Wang et al.	2005	China	1,000	Case-control	Increased risk of lung cancer with high alcohol intake.
Li et al.	2006	China	2,000	Cohort	No significant association between alcohol and lung cancer.
Zhang et al.	2007	China	1,500	Case-control	Increased risk of lung cancer with high alcohol intake.
Chen et al.	2008	China	1,200	Cohort	No significant association between alcohol and lung cancer.
Wu et al.	2009	China	1,800	Case-control	Increased risk of lung cancer with high alcohol intake.
Yang et al.	2010	China	1,600	Cohort	No significant association between alcohol and lung cancer.
Xu et al.	2011	China	1,400	Case-control	Increased risk of lung cancer with high alcohol intake.
Lin et al.	2012	China	1,300	Cohort	No significant association between alcohol and lung cancer.
Guo et al.	2013	China	1,700	Case-control	Increased risk of lung cancer with high alcohol intake.
Hou et al.	2014	China	1,900	Cohort	No significant association between alcohol and lung cancer.
Wang et al.	2015	China	1,100	Case-control	Increased risk of lung cancer with high alcohol intake.
Li et al.	2016	China	1,000	Cohort	No significant association between alcohol and lung cancer.
Zhang et al.	2017	China	1,200	Case-control	Increased risk of lung cancer with high alcohol intake.
Chen et al.	2018	China	1,300	Cohort	No significant association between alcohol and lung cancer.
Wu et al.	2019	China	1,400	Case-control	Increased risk of lung cancer with high alcohol intake.
Yang et al.	2020	China	1,500	Cohort	No significant association between alcohol and lung cancer.

10

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Oligo 7: 5' CCGGCCAGTC AGGCCACACT TAATTAAGTT TAAACGCGGC
 CCCGGCGCGC CTAGGTGTGT GCTCGAGGGC CCAACCTCAG
 TACCTGGTTC AGG 3'

Oligo 8: 5' CCGGCCTGAA CCAGGTACTG AGGTTGGGCC CTCGAGCACA
CACCTAGGCG CGCCGGGGCC GCGTTTAAAC TTAATTAAGT
GTGGCCTGAC TGG 3'

25

A plasmid derived from pRPA-RD-174 is obtained comprising other restriction sites between the

bromoxynil tolerance gene and the selectable marker kanamycin gene.

The schematic structure of the plasmid pRPA-RD-184 is represented in Figure 5 where the terms

5 "nos", "NPT II", "NOS pro", "35S pro", "BRX gene", "RB" and "LB" have the same meaning as in Figure 4.

pRPA-RD-235: Creation of an *Agrobacterium tumefaciens* vector containing the gene construct encoding thanatin
 10 **directed towards the extracellular matrix**

The plasmid pRPA-RD-232 is digested with the restriction enzymes *PmeI* and *AscI*, and the DNA fragment containing the gene for PR-la-thanatin is purified. The plasmid pRPA-RD-184 is digested with the same
 15 restriction enzymes. The DNA fragment containing the PR-la-thanatin expression cassette is then ligated into pRPA-RD-184. An *Agrobacterium tumefaciens* vector is thus obtained containing the sequence encoding the fusion protein PR-la-thanatin which leads to the
 20 expression of thanatin in the plant's extracellular matrix.

Example 2: Herbicide tolerance of transformed tobaccos

2.1 - Transformation

25 The vector pRPA-RD-235 is introduced into the *Agrobacterium tumefaciens* EHA101 strain (Hood et al., 1987) carrying the cosmid pTVK291 (Komari et al.,

1986). The transformation technique is based on the procedure of Horsch et al. (1985).

2.2 - Regeneration

The regeneration of the tobacco PBD6 (origin SEITA France) from foliar explants is carried out on a Murashige and Skoog (MS) basal medium comprising 30 g/l of sucrose as well as 200 µg/ml of kanamycin. The foliar explants are removed from plants cultivated in a greenhouse or *in vitro* and regenerated according to the foliar disc technique (Horsch et al., 1985) in three successive stages: the first comprises the induction of shoots on a medium supplemented with 30 g/l of sucrose containing 0.05 mg/l of naphthylacetic acid (NAA) and 2 mg/l of benzylaminopurine (BAP) for 15 days. The shoots formed during this stage are then developed for 10 days by cultivating on an MS medium supplemented with 30 g/l of sucrose but containing no hormones. Next, the developed shoots are removed and they are cultivated on an MS rooting medium with half the content of salts, vitamins and sugar and containing no hormone. After about 15 days, the rooted shoots are transferred into the soil.

2.3 - Bromoxynil tolerance

Twenty transformed plants were regenerated and transferred into a greenhouse for the pRPA-RD-235 construct. These plants were then treated in a greenhouse at the 5-leaf stage with an aqueous

suspension of Pardner corresponding to 0.2 kg of bromoxynil active material per hectare.

All the plants showing complete tolerance to bromoxynil are then used in various experiments which
 5 show that the expression of thanatin by the transformed plants makes them resistant to fungal attacks.

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